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Crypthecodinium cohnii with emphasis on DHA production: a review

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Abstract Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid (PUFA) that belongs to the ω-3 group. In recent years, DHA has attracted much attention because of its recognized beneficial effect on human health. At present, fish oil is the major source of DHA, but it may be produced by microorganisms with additional benefits. Marine microorganisms may contain large amounts of DHA and are considered a potential source of this important fatty acid. Some of these organisms can be grown heterotrophically on organic substrates without light, offering the possibility of greatly increasing microalgal cell concentration under controlled and monitored conditions, resulting in a very high quality product. Among the heterotrophic marine dinoflagellates, *Crypthecodinium cohnii* has been identified as a prolific producer of DHA. The organism is extraordinary in that it produces no other PUFAs than DHA in its cell lipid in any significant amount, which makes the DHA purification process very attractive, particularly for pharmaceutical and nutraceutical applications. This paper reviews recent advances in the biotechnological production of DHA by *C. cohnii*.

Keywords *Crypthecodinium cohnii* · Dinoflagellates · Docosahexaenoic acid (DHA) · Polyunsaturated fatty acids PUFAs

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Introduction

It is well known that both coronary heart disease and depression have exploded with Westernization, concomitant with changes in diet including declining intake of performed docosahexaenoic acid (DHA) (Joordens et al. 2007).

In fact, the role of DHA and other ω-3 long chain polyunsaturated fatty acids (LCPUFAs) in alleviating cardiovascular diseases has been shown in hundreds of in vivo and in vitro experiments apart from several clinical trials (Connor 2000). Both eicosapentaenoic acid (EPA) and DHA lower blood pressure and prevent the development of hypertension, one of the critical factors resulting in cardiovascular pathologies like atherosclerosis or stroke (Narayan et al. 2006).

In an experiment involving human subjects with hypertension and diabetes, it was found that higher DHA levels in blood platelet membranes resulted in significantly lower diastolic blood pressure and higher heart rate variability as compared to the control group, which had low DHA levels in the blood platelet membranes (Christensen et al. 2001). Li et al. (2003) stated that an adequate intake of ω-3 fatty acids including DHA at an early stage in life prevents increased blood pressure in later life in humans. In addition, data from intervention trials are consistent in suggesting that ω-3 LCPUFAs lower the risk of cardiovascular diseases, probably by the multiple mechanisms of lowering serum triacylglycerols, improving the LDL:HDL ratio, antiarrythmic effects on heart muscle, improved plaque stability, anti-thrombotic effects and reduced endothelial activation (Ruxton et al. 2005).

Compared to blood pressure, the etiology of cancer is complicated and multi-factorial apart from being uncertain. Nevertheless, a strong positive relationship between dietary fat intake and/or body fat and the manifestation of cancer

has been clearly established (Carroll 1986). DHA and EPA inhibit cyclocoxygenase, thereby reducing the amount of prostaglandins and increasing the lipoxygenase activity. This in turn results in higher production of hydroxyeicosatrienoic acids (HETE) and leukotriene B4 (LTB4), which have been suggested as retarding the process of cancerous cells taking over a tissue. In an experimental animal model, DHA was found to be more effective than EPA in inhibiting transcription factor activator protein 1 (AP-1), which has been implicated in the development of cancer (Liu et al. 2001).

In recent years, several case-control studies and clinical trials have shown that long chain ω-3 PUFAs, mainly EPA and DHA, play a very important role in neuropsychiatry performance. By effectively regulating the plasma/serum cholesterol that is associated with an increased risk of depression and suicide, these LCPUFAs aid in the prevention of neuropsychiatric disorders (Hibbeln and Salem 1995; Narayan et al. 2006).

The role of EPA and DHA in reducing the risk of neuropsychiatric disorders could be attributed to their effect on neurotransmitter receptor and G-proteins via effects on the biophysical properties of cell membranes and secondary messengers, and on protein kinases (Hibbeln and Salem 1995; Edwards and Peet 1999; Narayan et al. 2006).

A number of dietary-, ocular- (optical, retinal) and cerebral cortex-based factors have been suggested to influence the development of acuity; among the dietary factors is early postnatal intake of ω-3 LCPUFAs (SanGiovanni et al. 2000). In particular, DHA is regarded as essential for proper visual and neurological development of infants (Crawford et al. 1997; Das and Fams 2003; Nettleton 1992).

Maternal and preconceptional, prenatal and postnatal dietary balance and composition of essential fatty acids (EFA)/LCPUFAs influence the quantity of DHA available to the human fetus and milk-fed infant. Dietary DHA is more likely to be efficiently transferred from the mother to the fetus or young infant than the DHA that is synthesized from ω-3 EFAs (Crawford 1993). Therefore, dietary intake is the only practical means for the young infant to attain adequate LCPUFAs tissue status. In fact, the World Health Organization, the British Nutritional Foundation, the European Society of Pediatric Gastroenterology and Nutrition, and the International Society for the Study of Fatty Acids and Lipids have recognized the importance of DHA and arachidonic acid (AA) and recommended that LCPUFAs should be included in all infant formulas (Boswell et al. 1996). Presently, over 50% of all infant formulas in the United States contain a blend of DHA and AA (Wynn et al. 2005).

The actual recommended daily intake of DHA is 120 mg per day for men and 100 mg for women and 100 mg for aged people (Bourre 2005). Nevertheless, it is recognised that there is a lack of dietary ω-3 fatty acids in occidental countries. In order to prevent different pathologies, mainly cardiovascular diseases, and more lately some psychiatric disorders, an appropriate dietary level of ω-3 fatty acids is required, more specifically during development and aging. One way to meet this requirement is enriching food products (meat, butter, milk and dairy products, cheese and eggs, etc.) in ω-3 fatty acids (Bourre 2005). For instance, numerous animal feeding trials have been carried out using different species and breeds aiming at bringing the polyunsaturated fatty acid/saturated fatty acid (P/S) ratio of meat close to the recommended value (>0.7) (Raes et al. 2004). Bourre (2005) reported that by feeding animals with fish extracts or algae oils, the level of DHA was increased about 2-fold in beef, 7-fold in chicken, 6-fold in eggs, and 20-fold in fish (salmon). The author suggested that regulations governing the scheduling, raw materials and food programs should not just define minimum contents of toxic substances, but also define the real nutritional value of foodstuffs, which must nourish but not poison.

The traditional source of ω-3 fatty acids is fish oil. The major findings regarding this ω-3 source that received international recognition arose from reports from Danish scientists investigating the reasons why cardiovascular problems seemed nonexistent, or at least significantly less, in Greenland Eskimos compared to other populations in spite of the very high intake of fat by the Eskimos. A low incidence of heart desease in other fish-eating populations of Norwegians and Japonese also helped to focus attention on the importance of DHA and EPA being the two major PUFAs of fish oils (Ratledge 2005).

However, the use of fish oil as a food additive is limited due to problems associated with its typical fishy smell, unpleasant taste, and poor oxidative stability. Fish oil supply is difficult to predict, especially in the long term, with declining fish stocks in many of the world's oceans. In addition, the International Fishmeal and Oil Manufacturers Association estimates that inclusion of fish oil in aquaculture feeds will rise to 1,133,000 tons in 2010. This aquafeed demand in 2010 could result in a worldwide undersupply of fish oil. Moreover, the presence of EPA in fish oil is undesirable for application in infant food, as this fatty acid is associated with neonate growth retardation. In addition, serious questions remain about the possible contamination of fish oils with heavy metals and other pollutants (Carlson and Wilson 1994).

Microalgae biomass is particularly suitable for the extraction and purification of individual PUFAs due to its stable and reliable composition. In addition, PUFAs from cultured microalgae are cholesterol free, contaminant free [e.g. heavy metals, polychlorobiphenyls (PCBs)], and taste good (Medina et al. 1995). Van Beelen et al. (2007) com-

pared the mode of action of algal oils (including the microalga *Crypthecodinium cohnii*) and fish (menhaden) oil on cancer cells in vitro, and found similar results.

Attempts have been made to produce DHA phototrophically by growing microalgae in photobioreactors, but it is difficult to achieve high biomass concentration and high DHA productivity. This is due to unsolved problems, namely light limitation and oxygen accumulation, in photoautotrophic cultures (Jiang et al. 1999).

Large-scale production of algal fatty acids is possible through the use of heterotrophic algae using traditional fermentation systems which provide consistent biomass under highly controlled and monitored conditions resulting in a very high quality product. Fermentation runs last a matter of days, so production can be increased or decreased to match market requirements. In addition, there is no climatic or seasonal dependence. Hence, screening of microalgae for heterotrophic production of DHA is of potential significance. Among the heterotrophic marine dinoflagellate microalgae, *C. cohnii* was identified as a prolific producer of DHA. The organism is extraordinary in that it produces no other PUFAs other than DHA in its cell lipid in any significant amount (Van Pelt et al. 1999).

Habitat

Crypthecondium cohnii appears to be a species complex, composed of many reproductively isolated sibling species (Beam and Himes 1982). This microalga is brackish, littoral (Sournia 1986) and neritic (Himes and Beam 1975; Beam and Himes 1987). It is often present among macrophytes, particularly *Fucus* spp. (Ucko et al. 1997). Nevertheless, *Fucus* does not always yield *C. cohnii* (Beam and Himes 1977). In nature, this dinoflagellate also occurs among other decaying seaweeds (Ucko et al. 1997).

Crypthecodinium cohnii and its sibling species have also been isolated from other types of hosts like *Macrocystis*, *Sargassum* (Beam and Himes 1977, 1982), *Cystoseira*, *Zostera*, *Thalassia, Phragmites* and *Acetabularia* (Beam and Himes 1982).

Crypthecodinium cohnii-like dinoflagellates have been obtained from various habitats including open ocean beaches, polluted brackish bays and estuaries, steaming mangrove swamps and frozen New England harbors. Nevertheless, the strains of essentially globally dispersed dinoflagellates resembling *C. cohnii* are not members of the same gene pool (Himes and Beam 1978).

Crypthecodinium cohnii and *C. cohnii* sibling species are dispersed in temperate and tropical waters (Beam and Himes 1982). They are found from the North Sea to the Caribbean (Sournia 1986), and also in the Mediterranean Sea, the Baltic Sea, and the Atlantic, Pacific (Beam and Himes 1977) and Indian Oceans (Beam and Himes 1982).

The geographic distribution is difficult to interpret. Although the swarmers are motile, their speed (1 $km.y^{-1}$) must render their intrinsic vagility secondary to other agents of dispersion. They must have been affected by tides and currents, especially through rafting on fragments of macrophytes (Beam and Himes 1982).

Taxonomy

In 1753, the first modern dinoflagellates were described by Baker and named by Muller in 1773. The term derives from the Greek word δῖνος (dinos), meaning 'whirling,' and Latin flagellum, a diminutive term for a whip or scourge.

Dinoflagellates are an important group of phytoplankton in marine waters. These microorganisms are eukaryotes that have kept several prokaryotic features, and have some unique characteristics such as a permanent nuclear envelope, chromosomes condensed throughout the cell cycle and the lack of histomes and nucleosomes. The mitotic microtubular spindle is extranuclear, and passes through the nucleus via cytoplasmic channels (Moreau et al. 1998).

Dinoflagellate nomenclature is controversial. Traditionally, they were regarded as protozoans (one-celled animals) by some scientists and as one-celled algae (plants) by others. Currently, dinoflagellates are classified as Alveolates which are unicellular protists.

Crypthecodinium cohnii was described by Seligo (1885) as *Glenodinium cohnii*. Seligo provided good drawings showing the morphology of the cell with the characteristic cingulum, which is much displaced and does not form a complete loop around the body. Schiller (1933) transferred this organism to the genus *Gyrodinium* (Kofoid and Swezy 1921) as *Gyrodinium cohnii* (Seligo) Schiller. Biecheler (1952) described the new genus with the single species *Crypthecodinium setense* Biecheler, characterized by the presence of very thin thecal plates which could be demonstrated only by a special staining method. The cell morphology is very similar to that of *Gyrodinium cohnii*, and Biecheler (1952) already suspected the possible synonymy of the two organisms. Chatton (1952) regarded them as conspecific and introduced the combination *Crypthecodinium cohnii* (Seligo) Chatton. Since then, flagellates of this morphotype have been isolated several times and have been called *Crypthecodinium cohnii*. Ucko et al. (1989, 1997) reported a *C. cohnii*-like dinoflagellate occurring as a deleterious contaminant in commercial pond cultures of *Porphyridium* sp., a unicellular red alga, in Elat, Israel. Parrow et al. (2006) reported a heterotrophic dinoflagellate in a brackish-water fish aquarium at North Carolina State University, USA,

which was very similar to that described by Ucko et al. (1997) and fitted with Biecheler's description of *C. setense*. This study provided the first clear demonstration of the plate tabulation of a *Crypthecodinium* species, by scanning electron microscopy (SEM).

Crypthecodinium cohnii apparently represents a supra-species encompassing several biological species (Beam and Himes 1977; Beam et al. 1993).

Morphology

Two forms of *C. cohnii* have been reported, swimming cells and cysts (Bhaud et al. 1991) with different dimensions. The swimming cells show velocities of the order of 1 $km.y^{-1}$ (Beam and Himes 1982). The motile swimming cells have two unequal flagella. One is a flattened, ribbon-like flagellum, which encircles completely or partially the cell in a transverse groove, providing propulsive and spinning force for the cell. The other flagellum is directed posteriorly along a longitudinal groove and presumably acts like a rudder for steering. The flagella are inserted in two sulci (transverse-cingulum and longitudinal-sulcus, respectively) (Hackett et al. 2004). That part of the cell (whether cyst, thecate motile cell or athecate motile cell) anterior to the cingulum is termed the episome; the part of the cell posterior to the cingulum is termed the hyposome (Fensome et al. 1993). The schematic representation of *C. cohnii* by Perret et al. (1993) shows that the cingulum almost completely girdles the cell, as can be seen in Fig. 1. However, this schematic representation differs from the original description (Seligo 1885) in which the cingilum is much displaced and does not form a complete loop around the body (Ucko et al. 1997). Parrow et al. (2006) showed a scanning electron micrograph of a *Crypthecodinium* species in which the cingulum does not fully encircle the cell, traversing roughly two-thirds of the cell circumference (Fig. 2).

Crypthecodinium cohnii is a dinoflagellate with very delicate theca (15–20 μm) (Kubai and Ris 1969) whose plates are mainly composed of cellulose (Kwok and Wong 2003) and are barely visible, so it is sometimes necessary to use a special staining method (Ucko et al. 1997). During cell locomotion, the theca deforms (Sournia 1986). The theca is contained in relatively few alveoli with a pattern that can be determined (thecal plate tabulation) (Saldarriaga et al. 2004) and used for taxonomic purposes (Parrow et al. 2006).

The cysts are solitary and ovoid in shape (Sournia 1986) and can stay in a dormant/survival stage or start dividing (vegetative cysts) (Bhaud et al. 1991).

Ratledge et al. (2003) reported that the non-motile cysts contain greater levels of lipids, including DHA, than the swimmer form of the microalga.

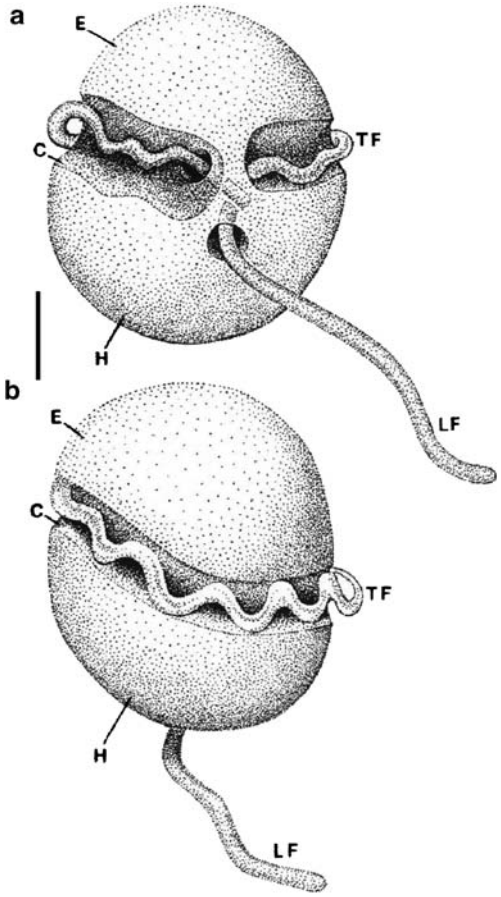

Fig. 1 Schematic representation of *Crypthecodinium cohnii* cell drawn from Perret et al. (1991). **A** Ventral view. **B** Dorsal view. *E* episome, *H* hyposome, *L.F.* longitudinal flagellum, *T.F.* transverse flagellum, *C* cingulum. *Bar* 5 μm. Reproduced with permission of the Company of Biologists

Reproduction

Crypthecodinium cohnii has both asexual and the sexual reproduction. It has been reported that the latter is induced by nutrient depletion (Tuttle and Loeblich 1975) and has been observed in dense, rapidly growing cultures (Ucko et al. 1997).

The vegetative cell cycle

Dinoflagellates are unicellular micro-organisms that are eukaryotes with a G1-S-G2-M cell cycle (Bhaud et al. 2000). In a normal cell cycle of the heterotrophic dinoflagellate microalga, motile G1 cells will shed their flagella, encyst and execute the remaining cell-cycle phases [DNA synthesis (S), G2 and mitosis (M)]. The cysts will subsequently produce 2, 4 or 8 daughter cells depending on which cell-cycle pathway the individual cells have taken (Wong and Whiteley 1996). Bhaud et al. (1994) determined the existence and lengthening of the G2-Phase (30 min) in

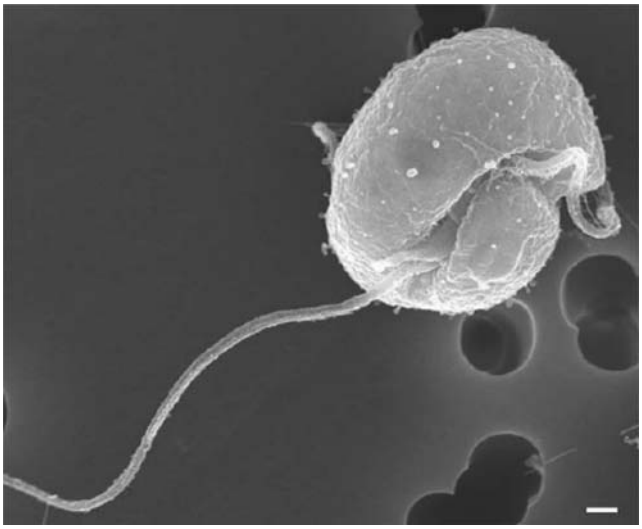

Fig. 2 Scanning electron micropgraph of a *Crypthecodinium* species showing the ventral view (from Parrow et al. 2006). Reproduced with permission of the Editor-in-Chief of the Afr. J. Mar. Sci.

the first cycle (cycle with swimming G1 phase) and the time of the second cell cycle phases (cycle in the cyst); G1, 30 min; S, 1.5 h; G2, 2 h and M, 2 h. These results, together with the estimation of the cell volume of the two and four swimming daughter cells emerging from the cysts allowed the authors to state the existence of two transition points: G1/S and G2/M, which are necessary for completion of mitosis (Fig. 3).

When investigating the biochemical or molecular biological events of the cell cycle in relation to the life cycle of the phytoplankton, it is imperative to have a synchronized population of cells. Wong and Whiteley (1996) described a method for synchronizing populations of *C. cohnii* UTEX 1649 using flow cytometric analysis of DNA content to follow the cell cycle progression of synchronized cells.

Kwok and Wong (2005) investigated the dynamics of lipid synthesis in the entire cell cycle of *C. cohnii*. The authors analyzed highly synchronized cells by flow cytometry using Nile Red-stained cells and observed a stepwise increase in polar lipid content and a continuous increase in neutral lipid content in the dinoflagellate cell cycle. They also determined the existence of a commitment point that monitors the synthesis of fatty acids at the late G_1 phase of the cell cycle.

Sexual development

Crypthecodinium cohnii sexual reproduction has isogamy and anisogamy forming a "fertilization bridge" (Bhaud et al. 1988). During fertilization, there is the protoplasmic fusion of gametes and the formation of the planozygote. The zygote encysts, producing the nuclear cyclose in the cyst (Ucko et al. 1997).

Crypthecodinium cohnii meiosis is peculiar as demonstrated by recombination of mobility mutants (Beam and Himes 1974, 1982; Tuttle and Loeblich 1974a, b; Himes and Beam 1975, 1978; Beam et al. 1977). Bhaud et al. (1991) reported either a conventional meiosis with two divisions and no crossing-over or a meiosis with only one division.

Ucko et al. (1997) reported that the gametes form groups of 3–8 cells which move in a lively manner around each other until two cells, anisogametes or isogametes, establish contact with their ventral sides and begin to fuse. The other flagellates swim away, whereas the fused pair does not move away but rotates. Then one of the two transverse flagella is shed, and the developing zygote, with two longitudinal flagella, swims away. Some time later the zygote encysts. The cysts divide and release normally only 2, but sometimes 4–8, dinoflagellates. The new dinoflagellates are motile as soon as they leave the cyst and are thophonts or gametes (Ucko et al. 1997).

C. cohnii fatty acid biosynthesis

Despite the importance of PUFAs, the pathways of formation in *C. cohnii* are still not well known (de Swaaf

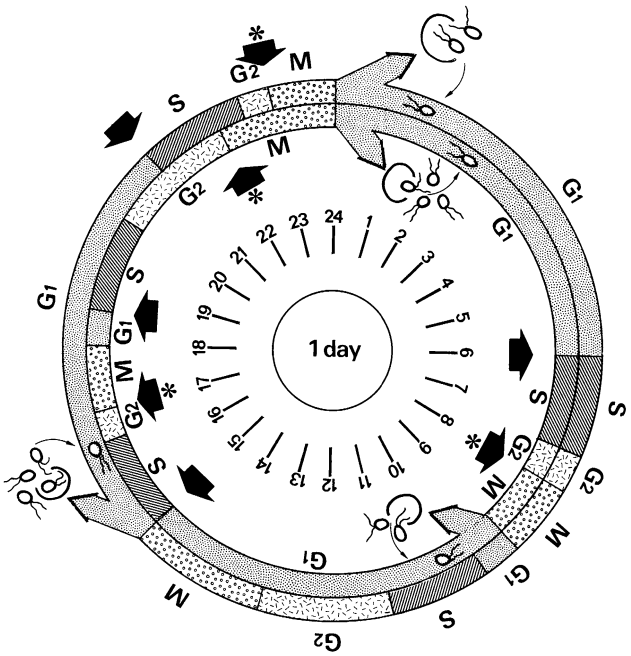

Fig. 3 Diagram of successive cell cycles of a dinoflagellate microalga over 24 h. In this particular example, one vegetative cell performed cell cycles (16 h) and released 4 daughter cells. One of these new swimming cells released 2 daughter cells 10 h later (*outer circle* of the diagram). During this time, other swimming cells gave an inverse alternation (*inner circle*). Different diagrams could be possible with other alternations. Transition points G1/S ("start" point) are presented by *arrows* and G2/M by *arrows plus star* (from Bhaud et al. 1994). Reproduced with permission of Wiley-Blackwell Publisher

et al. 2003a). The absence of chloroplasts offers a system for the study of the synthesis of PUFAs without the involvement of chloroplast glycolipids (Henderson et al. 1988). In fact, this microalga has been used to study the pathways involved in PUFA synthesis by marine microalgae (Beach et al. 1974; Sonnenborn and Kunau 1982; Henderson and Mackinlay 1991). *Crypthecodinium cohnii* can accumulate lipid to over 20% of dry weight with a high content of 22:6ω-3 [over 30% of total fatty acid (TFA) content]. Other PUFAs remain below 1% of the TFA content (Harrington and Holz 1968; Beach and Holz 1973; de Swaaf et al. 1999). This is remarkable as most marine microalgae rich in PUFAs contain intermediate fatty acids in the cascade of elongation and desaturation, as the saturated fatty acids are successively desaturated and elongated through a series of reactions, using the fatty acid synthetase (FAS) complex of enzymes, leading to the formation of various intermediate PUFAs. Therefore, it has been suggested that the final step leading to *C. cohnii* DHA production is distinct from that found in most microorganisms (Meyer et al. 2003; Ratledge 2004).

So far it has not been clear whether desaturases are involved in *C. cohnii* DHA production (de Swaaf et al. 2003a). In fact, Sonnenborn and Kunau (1982) reported in vitro production of saturated fatty acids, mainly 14:0 and 16:0, by a purified cytosol enzyme complex of *C. cohnii*. They concluded that the FAS system should be operative in *C. cohnii* and might supply the precursors for DHA biosynthesis. However, Beach et al. (1974) incubating *C. cohnii* cells in the presence of ^{14}C-labeled fatty acids ranging from 10 to 18 carbon atoms found that label was detected in oleic acid (18:1ω9, the most abundant monounsaturated fatty acid in *C. cohnii*) but not in DHA. In contrast, Henderson and Mackinlay (1991) detected a small amount of label in 22:6ω-3 when the microalga was grown in the presence of ^{14}C labeled 18:0 or 18:1. De Swaaf et al. (2003a) tested the ability of *C. cohnii* to use ^{13}C-labeled externally supplied precursor molecules for 22:6 biosynthesis by ^{13}C NMR analysis and studied the presence of desaturases (typical for aerobic PUFA synthesis). They suggested that the fatty acid production by *C. cohnii* may be explained by the presence of three tightly regulated separated systems, namely (1) the biosynthesis of saturated fatty acids, (2) the conversion of saturated fatty acids to monounsaturated fatty acids, and (3) de novo synthesis of 22:6ω-3 with desaturases involved, although it is not clear whether oxygen-dependent desaturases are active in the biosynthesis of DHA in *C. cohnii*. However, the system appears to be aerobic as, in the study of Beach and Holz (1973), the DHA and monounsaturated fatty acids sharply decreased when a growing culture of *C. cohnii* was switched from gassing with air to nitrogen gas.

Ratledge (2004) suggested that DHA biosynthesis in *C. cohnii* could use the PKS route, similar to that found in *Schizochytrium* sp. and probably related thraustochytrid marine protists. In these DHA-synthesising thraustochytrid organisms, the growing fatty acyl chain is not reduced to completely saturated fatty acids as occurs with the "conventional" eukaryotic FAS system, but involves fatty acyl intermediates that remain unsaturated as the chain continues to be lengthened. If this is found to be the case, it would help to explain why it has always been difficult to identify conventional, eukaryotic-like desaturases in *C. cohnii* (Ishiwaka et al., unpublished work referred by Ratledge 2004).

Nutrition

Crypthecodinium cohnii is an obligatory heterotrophic marine dinoflagellate. This implies that it has the capacity for sustained growth and cell division in the dark. Free energy and cell carbon are both obtained from the metabolism of an organic carbon substrate. This and other nutrients are obtained from living or decomposing seaweeds from which they are normally recovered. It has been suggested that *C. cohnii* might have parasitic capabilities in or on macrophytes, as motile cells of all strains appear to have a peduncle, an organelle employed by other dinoflagellates on phagotrophy (Beam and Himes 1987).

Some strains of *C. cohnii* are phagotrophic, like the Elat strain, which preys on cells of the red microalga *Porphyridium* sp. The food uptake of *C. cohnii* Elat strain is a typical myzocytosis, in that the cell content of the prey is ingested by a feeding tube, leaving the prey's cell coat in the medium (Ucko et al. 1997).

Cultivation

Crypthecodinium cohnii has had a long history of laboratory cultivation dating back to early twentieth century (Kyle 1996). *Crypthecodinium cohnii* stands out as a model free-living species complex that can be cultured using both synthetic liquid and solid media (Tuttle and Loeblich 1975; Beam and Himes 1980; Bhaud et al. 1991), likely because of an inherent inclination for resorption related to its natural occurrence amid rotting seaweed (Parrow and Burkholder 2003).

Carbon sources

Originally *C. cohnii* was grown in enriched seawater broths (Tuttle and Loeblich 1975). In a medium containing yeast

extract, sodium acetate and peptone in sea water, *C. cohnii* can readily be propagated in the laboratory (Pringsheim 1956).

Crypthecodinium cohnii strains grow well on organic carbon substrates such as glucose (strain Seligo, Tuttle and Loeblich 1975; strain ATCC 40750, Kyle et al. 1998; strain UTEX L 1649, Vazhappilly and Chen 1998; strains ATCC 30556, ATCC 50051, UTEX L 1649 and RJH, Jiang et al. 1999; strains ATCC 30556, ATCC 50051 and RJH, Jiang and Chen 1999; strain ATCC 30772, de Swaaf et al. 1999; strains UTEX L 1649, CCMP 316 and ATCC 50297, Vazhappilly 1999; strain ATCC 30556, Jiang and Chen 2000a; strain ATCC 30556, Jiang and Chen 2000b; strain ATCC 30772, de Swaaf et al. 2003c), dextrose (strain ATCC 40750, Kyle 1996), ethanol (strain ATCC 30772, de Swaaf et al. 2003b; strain ATCC 30772, Sijtsma and de Swaaf 2004), acetic acid (strain ATCC 30772, Ratledge et al. 2001a; strain ATCC 30772, de Swaaf et al. 2003c; strain ATCC 30772, Sijtsma and de Swaaf 2004), sodium acetate (strain UTEX L1649, Vazhappilly and Chen 1998; strain ATCC 30772, Ratledge et al. 2001a), and carob pulp syrup (strain CCMP 316, Mendes et al. 2007a).

According to some authors, *C. cohnii* grows on glycerol (Beam and Himes 1980). However de Swaaf et al. (1999) reported that no or marginal growth (less than one doubling) was observed when glycerol was offered as a carbon source. *Crypthecodinium cohnii* ATCC 30772 was able to grow on galactose and no or marginal growth was observed on sucrose (de Swaaf et al. 1999). *Crypthecodinium cohnii* was unable to grow on fructose, maltose, rhamnose, arabinose, lactose, galacturonic acid, pectina and aldose (Beam and Himes 1980). Disagreeing with this, the patent by Takeuchi et al. (1994) claims that fructose and galactose can be assimilated by *C. cohnii* ATCC 40750 as carbon sources. According to the patent of the Kawasaki Steel Corporation (Borowitzka 1995), *C. cohnii* can also be incubated in a medium containing fish oil, soybean oil or lactate as a carbon source. However, glucose is the most commonly used substrate for microbial lipid production (Singh and Ward 1996; Sijtsma et al. 2005).

Nitrogen sources and other nutrients

In addition to a reduced carbon source, a nitrogen source must be provided to the culture medium [e.g., peptone, yeast extract (YE), meat extract, glutamic acid, waste molasses, and corn steep liquor, KNO_3, NH_4Cl] (Borowitzka 1995). Takeuchi et al. (1994) also refer to the use of urea, meat casein hydrolysate and $(NH_4)_2SO_4$ as nitrogen sources. With regard to inorganic salts, natural seawater may be the best, but various types of known artificial seawater may also be used, as well as various sodium salts, phosphates, magnesium salts, potassium salts, borates and carbonate salts. Also useful are trace amounts of heavy metals such as iron salts, manganese salts, cobalt salts, zinc salts, chlorine compounds and bromine compounds (Takeuchi et al. 1994; Borowitzka 1995). Therefore, *C. cohnii* can grow in culture media containing inexpensive nutrients, which can be used in industrial bioprocesses.

Growth

Provasoli and Gold (1962) prepared the first synthetic medium to culture *C. cohnii* which was a great improvement over earlier preparations since it eliminated many unnecessary ill-defined components. Later, Tuttle and Loeblich (1975) experimentally defined a synthetic medium, MLH, for optimal growth of this dinoflagellate.

Trying to discover optimal growth conditions for *C. cohnii*, Jiang et al. (1999) tested three previously reported media: Porphyridium medium (Jiang and Chen 1999), A_2E_6 medium (Nerad 1993) and *C. cohnii* medium (Spector 1984), all with glucose as the principal carbon source, and concluded that the *C. cohnii* strains studied show best growth and highest specific grow rate on Porphyridium medium. The authors also investigated the effect of medium glucose concentration on growth over a range of glucose concentrations (5–40 g L^{-1}) in Porphyridium medium. The highest specific growth rate, highest cell dry weight concentration and highest growth yield on glucose were obtained at 20 g L^{-1} glucose. The lower specific growth rate and cell dry weight observed at higher glucose concentrations were probably due to glucose inhibition (Jiang and Chen 2000a). De Swaaf et al. (1999) also reported glucose inhibition above 25 g L^{-1} in *C. cohnii* shake flask cultures. However, *C. cohnii* was able to grow on a glucose concentration as high as 84.3 g L^{-1} in a 1-L bioreactor (de Swaaf et al. 1999).

With regard to nitrogen and micronutrients sources, de Swaaf et al. (1999) reported an optimum yeast extract concentration of 2 g L^{-1} for growth of *C. Cohnii* in the studied concentration range of 0–10 g L^{-1}.

De Swaaf et al. (2003b) also studied the influence of ethanol concentration on growth of *C. cohnii* shake flask cultures grown on a complex medium containing yeast extract and sea salt. The specific growth rate was optimal with 5 g L^{-1} ethanol and growth did not occur at 0 g L^{-1} and above 15 g L^{-1}, indicating that higher ethanol concentrations inhibited *C. cohnii* growth.

Vazhappilly and Chen (1998) concluded that *C. cohnii* UTEX L1649 showed better heterotrophic shake flask growth on glucose (5 g L^{-1}) than when acetate (1 g L^{-1}) was used as the sole carbon and energy source. However, laboratory-scale, pH controlled, fed-batch cultivations of *C. cohnii* ATCC 30772, a so-called pH auxostat culture with

50% acetic acid as carbon source, showed higher biomass concentration than when glucose was used as the main carbon source (de Swaaf et al. 2003c).

Temperature

The optimum temperature of *C. cohnii* is 27°C (Ishida 1968; Tuttle and Loeblich 1975). Jiang and Chen (2000b) studied the effect of temperature in *C. cohnii* ATCC 30556 and observed that the microalga grew well over the entire range of studied temperatures, 15–30°C. These results are in good agreement with the previously reported cell growth inhibition when the culture temperature was below 14°C or above 31°C (Beach and Holz 1973). Gold and Baren (1966) reported an optimum temperature of 35°C for a *C. cohnii* strain isolated from Puerto Rican water and a growth inhibition at 20°C and 30°C. According the cultivation process patented by the Martek Corporation (Kyle et al. 1991), cultivation of *C. cohnii* ATCC 40850 can be carried out at any life-sustaining temperature. Generally, *C. cohnii* grows at temperatures ranging from 15 to 34°C. Strains which grow at higher temperatures are preferred for industrial applications (Kyle 2004). For large scale cultivation, a higher cultivation temperature is also desirable, due to an improved cooling capacity at higher temperatures (de Swaaf et al. 1999).

Oxygen supply

As *C. cohnii* is an obligate aerobic organism, cultivation requires an efficient oxygen transfer from gas phase to culture broth. In shake flask cultivation, a reciprocal shaker is sufficient, but in bioreactors an oxygen-efficient supplier is necessary. Growth of *C. cohnii* ATCC 30772 was improved significantly by enhancing the agitation speed from 50 to 100 rpm in shake flask cultivation (OD increased more than four times) (de Swaaf et al. 1999). These results are in good agreement with those reported by Beach and Holz (1973) who reported that the microalgal cells with an enhanced supply of O_2, from shake flask and bioreactor cultures, multiplied more rapidly than cells grown under a restricted supply of oxygen. In *C. cohnii* bioreactor cultivations, oxygen levels should be maintained at a dissolved oxygen level of, at least 10% of air saturation (Kyle et al. 1995, 1998).

Although it has been reported that mechanical agitation has specific growth inhibition effects on dinoflagellates (Berdalet 1992; Berdalet and Estrada 1993; Thomas and Gibson 1990, 1992; Yeung and Wong 2003), relatively high speeds have been successfully used in *C. cohnii* ATCC 30556 shake flask (150 rpm; Jiang and Chen 2000a, b) and in *C. cohnii* ATCC 30772 bench bioreactor cultivations (1,250 rpm; de Swaaf et al. 2003b, c). De Swaaf et al. (2003c) reported that, in a *C. cohnii* high-cell-density fed-batch cultivation for DHA production, vigorous mixing was required to sustain a sufficient oxygen level during high microalgal cell density cultivation. This was complicated by increasing culture viscosity, which resulted from the production of viscous extracellular polysaccharide. However, the viscosity of the culture supernatant was strongly reduced when a commercial polysaccharide-hydrolase (Glucanex®) was added to the *C. cohnii* high-cell-density fed-batch culture.

pH

The optimum pH for *C. cohnii* was 6.6 in the range 5.2–7.0 tested (Tuttle and Loeblich 1975). However, the cultivation can be carried out over a broad pH range, typically from about 5.0 to 9.0. Preferably, a pH range from 6.0 to 7.0 is used for the growth phase (Kyle et al. 1991). Jiang and Chen (2000a) studied the effect of initial medium pH (varying from 4 to 10) on growth of *C. cohnii* and obtained the highest specific growth rate, highest cell dry weight concentration and highest growth yield on glucose when the medium pH was 7.2.

Salinity

Jiang and Chen (1999) studied the effect of salinity on cell growth of three marine strains of *C. cohnii* (ATCC 30556, ATCC 50051, RJH) in shake flask cultures and observed that lag phases of the three strains increased with increasing salinity in Porphyridium medium. Optimum NaCl concentration for cell growth is strain specific (Jiang and Chen 1999). According to de Swaaf et al. (1999), for *C. cohnii* ATCC 30772 strain optimal growth, a minimal sea salt concentration of 17.8 g L^{-1} is required, which is about half the average sea water salinity. The observation of growth inhibition at low salinity is in agreement with previous data. Inhibition of growth at low (<5 g L^{-1} NaCl) and also high salinity (>50 g L^{-1} NaCl) has been reported in shake (Beach and Holz 1973) and standing (Tuttle and Loeblich 1975) cultivations of *C. cohnii*. For large-scale cultivation processes, the salt concentration should preferably be as low as possible in order to prevent corrosion problems (de Swaaf et al. 1999). Behrens et al. (2005) patented methods of increasing production of highly unsaturated fatty acids by marine microorganisms, including *C. cohnii*, growing in low chloride media by manipulation of sodium and potassium ion levels. The authors have identified culture conditions that allow *C. cohnii* to be grown in medium with substantially lowered chloride levels without adversely affecting dry weight, fat content or DHA

content when compared to growth in a normal "high chloride" medium, in order to prevent corrosion in stainless steel fermenters.

Light

Tuttle and Loeblich (1975) reported that *C. cohnii* WH-d grows fastest in the dark. The authors observed that cells grown in fluorescent light of 17.9 $W.m^{-2}$ contained 4.5 times more carotenoids than dark-grown cells. The authors suggested that this increase in pigment production could account for the decreased growth rate in the presence of visible light due to increased anabolic energy going to protective pigments rather than to cellular division.

Composition and metabolites

Henderson et al. (1988) reported that lipids accounted for 24.6% of the dry weight of *C. cohnii* WH-d cultured for 6 days to stationary phase in shake flasks containing MLH medium. These lipids were mostly neutral lipid (71.6%, w/w) of which triacylglycerols (TAG) were the major component (76.4%, w/w). Phosphatidylcholine (PC) was the major polar lipid (63.6%, w/w). Phosphatidylethanolamine (PE) was the second most abundant phospholipid although it was present in very much smaller amounts than PC. TAG fatty acids were predominantly saturated, whereas 57% (w/w) of the fatty acids in PC were polyunsaturated. 22:6ω-3 accounted for over 50% of the fatty acids in PC. Therefore, the DHA in *C. cohnii* WH-d was found to be mostly distributed in PC, the main polar lipid fraction. Beach and Holz (1973) and Jiang and Chen (2000b) supported this observation. On the other hand, Wynn et al. (2005) stated that DHA accumulates predominately in *C. cohnii* cells as TAG, the neutral lipid fraction. In addition, the industrial process patented by the Martek Coporation reported that *C. cohnii* biomass extractable oil comprized more than c. 70% triglycerides having, in general, 30–50% DHA (Kyle 2004). These differences in DHA in the different lipid classes reported by different authors may be due to differences in age culture when the microalgal cells were harvested, cultivation conditions and strains that were used.

Studying the effect of aeration on *C. cohnii* cell fatty acid composition, Beach and Holz (1973) reported that, in fermenter cultures gassed with air, the major neutral lipid fraction, TAG (44% of neutral lipids), contained: 22:6n−3 25%, 18:1n−9 13%, 16:1 1%, 14:1 <1%, 18:0 1%, 16:0 25%, 14:0 28%, and 12:0 10.01%. The major polar lipid fraction, PC (79% of polar lipids), contained 22:6n−3 66%, 22:5n−3 <1% 18:1n−9 2%, 18:0 4%, 16:0 18%, and 14:0 9%. Gassing with N_2 caused a rapid and marked elevation of 12:0 and 14:0, and to a lesser extent 10:0 and 18:0, and a depression of 18:1n−9, 16:0 and 16:1 and 22:6n−3 in TAG. Variations in temperature and in salinity caused only minor compositional changes in TAG. None of the environmental perturbations significantly changed the fatty acid composition of the PC.

Similar to plant cells, cellulose is the major constituent of the *C. cohnii* cell wall (Kwok and Wong 2005). *Crypthecodinium cohnii* has no photosynthetic pigments but, as a reserve, stores plenty of starch and lipids (Pringsheim 1956; Loeblich 1976). Photosynthesis-associated lipids (mono- and digalctosyldiglycerids, and sulfoquinovosyl diglyceride and their polyunsaturated C_{18} and C_{20} fatty acids) are not present. Carotene concentration is higher in light-grown cells than in dark-grown ones (Tuttle et al. 1973), but fatty acid composition is not different (Holz and Beach 1980).

The lipids of *C. cohnii* include isoprenoid types (β-carotene and γ-carotene) (Tuttle et al. 1973) responsible for the yellow-orange color of DHA-rich oil extracted from the alga, 4α-methyl sterols, dinosterol, dehydrodinosterol (4α,23,24-trimethylcholesta-5,22-dien-3β-ol) and the tentatively identified 4α,24-dimethyl- cholestan-3β-ol and 4α,24-dimethylcholest-5-en-3β-ol. The major 4-demethyl sterol was cholesta-5,7-dien-3β-ol which was accompanied by a smaller amount of cholesterol and traces of several other C_{27},C_{28} and C_{29} sterols. In addition, a 3-oxo-steroid fraction was isolated and the major component identified as dinosterone (4α,23,24-trimethylcholest-22-en-3-one) (Withers et al. 1978).

De Swaaf et al. (2001) characterized extracellular polysaccharides (EPS) produced by *C. cohnii*. The crude EPS contained 55% polysaccharides, 10% proteinaceous components, 10% unknown material (precipitate after hydrolysis), 7% ash and trace amounts of Si, Cl, Fe, Zn and Cu. *C. cohnii* produced multiple EPSs, variable in size and sugar composition. A high molecular mass fraction (from 100 kDa to >1,660 kDa) and a medium molecular mass fraction (6–48 kDa) were detected. The high molecular mass fraction contained (on a molar basis) 71.7% glucose, 13.1% galactose and 3.8% mannose, whereas the medium molecular mass fraction contained 37.7% glucose, 19.8% galactose and 28.1% mannose. Other monomers present in both fractions were fucose, uronic acid and xylose.

DHA production

Cultivation scale and volumetric productivity (r_{DHA}) have been identified as major factors in determining the economic feasibility of fermentative DHA production (Sijtsma

et al. 1998). Factors that determine r_{DHA} are biomass concentration, lipid content of the cells, DHA content of the lipid and cultivation time (de Swaaf et al. 2003c). For economically feasible industrial cultivations of *C. cohnii*, high cell densities are required so it is necessary to optimize the culture medium providing basic data for commercial production of DHA. Growth and fatty acid formation are affected by key medium components and environmental conditions, and a large number of studies have been published, aiming at the DHA production optimization.

Carbon sources

In order to find suitable carbon sources for *C. cohnii* lipid accumulation, different carbon sources were studied by de Swaaf et al. (1999). After 50 h incubation, the lipid content of cells grown on glucose was 13.4% (w/w) whereas cells grown on galactose contained less 11.4% (w/w). The DHA content of the lipids was similar (35.2% with glucose and 36.2% with galactose).

Jiang and Chen (2000a) studied the effect of medium glucose concentration on the DHA content of *C. cohnii*. Although low glucose concentrations enhanced the degree of fatty acid insaturation and DHA formation, this was accompanied by a slow growth rate. Other studies carried out by de Swaaf et al. (2003b, c) investigated the effects of different carbon sources (glucose, acetic acid and ethanol) on *C. cohnii* biomass, lipid, and DHA concentrations. Lipid concentration was found to decrease when cell growth was exponential but, subsequently, increased exponentially when growth was linear owing to an undefined nutrient limitation. Therefore, it appears that lipid production occurs under growth-limiting conditions; during linear growth, the cells are stressed due to nutrient limitation and therefore produce more lipids. It also appears that the quality of the lipid (in % DHA) is affected by growth rate and lipid concentration—the concentration of DHA is negatively affected by increases in lipid concentration. The highest quality lipid (in % DHA) was obtained when glucose was used as the carbon source, and when the cell concentration and lipid content of the cells were the lowest. These results show that optimum growth and maximum DHA accumulation by the microalgae cells require different medium compositions.

De Swaaf et al. (1999) studied the DHA production by *C. cohnii* ATCC 30772, using glucose as the carbon source. In these experiments, the total amounts of lipid and DHA after 91 h attained 3.7 and 1.6 g L^{-1}, respectively (Table 1). However, compared with glucose, the use of acetic acid and ethanol as carbon sources proved to be much more efficient with respect to DHA production (Ratledge et al. 2001a; de Swaaf 2003, b, c; Table 1). Laboratory-scale, pH controlled, fed-batch cultivations of *C. cohnii* ATCC 30772, a so-called pH auxostat culture with 50% acetic acid as the carbon source, achieved lipid and DHA concentrations of 28 and 8 g L^{-1} respectively, after 210 h (Ratledge et al. 2001a; de Swaaf et al. 2003c). The use of acetic acid/acetate as the carbon source does not require the imposition of a stationary phase in order to obtain satisfactory production of DHA. This is particularly advantageous as it will facilitate adaptation of the method to a continuous or semi-continuous process (Ratledge et al. 2001a).

In comparison with several other *C. cohnii* strains, ATCC 30772 appeared to be the best strain with respect to DHA production using acetate/acetic acid as carbon sources (Ratledge et al. 2001a). The productivity of DHA by this strain was even further increased by the use of pure acetic acid and prolonged cultivation periods (de Swaaf et al. 2003c). This resulted in cultures producing 61 g lipid L^{-1} and 19 g DHA L^{-1} after 100 h of cultivation (Table 1). The maximum overall productivities of lipid and DHA were 152 mg L^{-1} h^{-1} and 48 mg L^{-1} h^{-1}, respectively.

A further improvement was achieved by the development of an ethanol fed-batch protocol (de Swaaf et al. 2003b). In a fed-batch cultivation of *C. cohnii* ATCC 30772 with pure ethanol as feed, 83 g dry biomass L^{-1}, 35 g lipid L^{-1} and 11.7 g DHA L^{-1} were produced in 220 h. The overall volumetric productivity of DHA in this process was 53 mg L^{-1} h^{-1}, the highest value ever reported for this alga (Table 1).

Despite the high DHA productivities obtained from these feedstocks, acetic acid in large-scale fermentations requires careful handling, as any spillage or contact with the skin must be dealt with promptly. Moreover, high capital investment and maintenance costs should be considered for fermentation hardware when dealing with a corrosive feedstock such as acetic acid. Ethanol flammability makes

Table 1 DHA productivities of *Crypthecodinium cohnii* on different carbon sources (DCW = Dry cell weight)

Strain	Fermentation time (h)	Carbon source	Cell concentration (g DCW L^{-1})	DHA concentration (g L^{-1})	DHA productivity (mg L^{-1} h^{-1})	Reference
C. cohnii ATCC 30772	91	Glucose	27.7	1.4	19	de Swaaf et al. (1999)
C. cohnii ATCC 30772	400	Acetic acid	109.0	19.0	48	de Swaaf et al. (2003c)
C. cohnii ATCC 30772	220	Ethanol	83.0	11.7	53	de Swaaf et al. (2003b)
C. cohnii CCMP 316	100	Carob pulp syrup	42	1.9	18.5	Mendes et al. (2007a)

this feedstock unsuitable when stored and transported around a production site in its undiluted form. In addition, processes using ethanol may need to be continuously scrutinized by regulatory authorities to prevent the use of the ethanol for purposes other than for which it is intended. This may place unwanted restrictions on its suitability as a large-scale fermentation feedstock (Sijtsma et al. 2005). Therefore, alternative handling carbon sources for *C. cohnii* DHA production should be found. Mendes et al. (2007a) reported a successful *C. cohnii* fed-batch fermentation for DHA production using diluted carob pulp syrup (1:10.5 v/v) supplemented with yeast extract and sea salt attaining 42 g dry biomass L^{-1} and 1.9 g DHA L^{-1} after 100 h (Table 1).

Growth phase

The growth phase also influences DHA production. Jiang and Chen (2000b) reported that, as the culture aged up to the early stationary phase, the DHA content increased. The changes in fatty acid composition, such as the DHA content increase and the decrease in saturated fatty acid content in the late exponential phase or early stationary phase, might be the result of complete consumption or starvation of some specific nutrients in the medium that induced qualitative and quantitative changes in fatty acids. Sufficient nutrient supply might lead to the synthesis of more saturated fatty acids. On the contrary, nitrogen limitation might result in the formation of more unsaturated fatty acids (Chen and Johns 1991). Therefore, industrial *C. cohnii* fermentations are usually a carbon-fed batch process and progress in two stages. The first is the active growth phase under nutrient excess conditions, during which the lipid content of the biomass is modest (approximately 20% w/w dry wt). Once the nitrogen source is depleted, carbon is continuously supplied to the fermenter. Since cell growth and division is halted due to the lack of nitrogen for de novo protein and nucleotide synthesis, the supplied carbon is converted into a storage lipid (TAG) rich in DHA (Wynn et al. 2005). During this lipid accumulation phase, *C. cohnii* cells lose their flagella and become "cyst-like" cells packed with DHA-rich lipid bodies. Maintaining the carbon concentration in the cultivation vessel is important to optimize lipid accumulation not only to promote synthesis but also to avoid utilization of the internal storage lipids (Wynn et al. 2005).

Salinity

The effect of salinity on docosahexaenoic acid production by *C. cohnii* was studied by Jiang and Chen (1999) in shake flask cultures. They concluded that, despite the optimum NaCl concentration for DHA production being strain specific, all strains responded to the change of salinity by modifying their cellular fatty acid compositions. The content of total fatty acids did not show an obvious correlation to salinity, but was decreased when the culture was at a higher salinity. Beach and Holz (1973) also reported that the proportion of DHA decreased when the salinity was above 30 g L^{-1}. De Swaaf et al. (1999) reported that growth and lipid accumulation were stimulated by increasing salinity in the range 2.8–27.8 g L^{-1} studied.

Temperature

The effects of temperature and temperature shift on the fatty acid composition, DHA content and productivity of *C. cohnii* ATCC 30556 were investigated by Jiang and Chen (2000b). They concluded that higher temperatures (over the range 15–30°C) favored growth with the highest specific growth rate of 0.092 h^{-1} at 30°C. In contrast, low temperature favored the formation of polyunsaturated fatty acids. In fact, cells grown at low temperatures adapted themselves by increasing the proportion of unsaturated fatty acids, particularly polyunsaturated fatty acids, in order to maintain proper membrane lipid fluidity and functions (Jiang and Chen 2000b). The highest DHA content was obtained at 15°C in the early stationary phase (72 h) (Jiang and Chen 2000b). De Swaaf et al. (1999) also reported that lipid accumulation was clearly influenced by the incubation temperature. When *C. cohnii* ATCC 30772 cells were grown at 27°C for 50 h, the lipid content was 13% compared to 7.8% obtained at 30°C. However, regarding the DHA percentage of lipids, at 27°C it was 35.9%, while at 30°C it attained 40.4%. Beach and Holz (1973) found a higher lipid content in *C. cohnii* cells grown at 31.5°C (11%) than at 25°C (8.7%). These authors, however, harvested the cultures grown at 31.5 and 25°C at different cultivation times (after 4 and 2 days, respectively).This difference in time of harvest may strongly affect the lipid production.

Biocompatible organic solvents

Biocompatible organic solvents have been used in milking microlagae to extract products such as β-carotene from *Dunaliella salina* in two phase bioreactors, leading to higher productivity (Hejazi and Wijffels 2004). A similar process was used for DHA production enhancement from *C. cohnii* (Silva et al. 2006). In this work, two *C. cohnii* carbon-fed batch fermentations were carried out simultaneously in the presence and absence of n-dodecane. It was found that the DHA content of biomass, DHA percentage of TFA and DHA volumetric rate increased 10 and 47%, respectively, compared to the fed-batch fermentation without n-dodecane. It was suggested that the higher amounts of DHA obtained in the n-dodecane presence were produced by substitution of extracted lipids by newly synthesized lipid, enhancing the

overall productivity of the system. The n-dodecane presence also resulted in higher dissolved oxygen tensions and microalgal biomass concentrations, compared to the fermentation with no n-dodecane.

DHA industrial production and patents

Using classical strain screening, many microalgae strains have been isolated that have potentially attractive attributes as DHA producers. Screening for increased lipid production and improved growth under conditions suitable for large-scale production have also been sucessfully accomplished before scale-up trials by many researchers, as described above.

Successful cultivation of *C. cohnii* to produce commercial edible oil containing DHA has been achieved by Martek Corporation in Maryland, USA. Martek has developed a GMP process utilizing a wild type *C. cohnii* growing on glucose as the main carbon source to produce single cell oil (DHASCO) with a DHA enrichment of greater than 40% (Kyle 1996). Cultures of the organism were grown in liquid medium in shake flasks and transferred to progressively larger vessels. Temperature, pH, air flow, pressure, agitation and dissolved oxygen were all continuously monitored and controlled. When the culture reaches a specific cell density, production of single cell oil was induced by imposition of stationary phase (e.g., by nitrogen depletion or a pH rises) (Kyle et al. 1998). It was the carbon source to nitrogen source ratio which promoted the efficient production of the single cell oil (Kyle et al. 1995, 1998). Using glucose and yeast extract as exemplary, a preferred ratio of carbon source to nitrogen source is about 10–15 parts of glucose to 1 part yeast extract (Kyle et al. 1998).

It is known that, among culturing methods of microorganisms, batch and fed-batch culture are not effective in maintaining a high productivity due to the accumulation of secondary metabolites and waste material which inhibit microbial growth. The Kawasaki Steel Corporation patented a process for producing *C. cohnii* in a high cell density by continuous culturing. The continuous culture was carried out by controlling medium sugar (glucose) at a certain level, preferably from 0.2 to 3.0% w/w. Yeast extract was used as a nitrogen source at from one-fifth to one-tenth of glucose concentration. The dilution rate of the supply medium should be below 0.25 h^{-1}, preferably 0.1–0.2 h^{-1}, to obtain the maximum productivity (Takeuchi et al. 1994).

In Japan, several heterotrophic processes for producing docosahexaenoic acid by *C. cohnii* have been patented (Borowitzka 1995).

Ratledge et al. (2001b) patented a method in which *C. cohnii* is cultured in a suitable growth medium with acetic acid/acetate as the main carbon source. The acetate is provided, and replenished, by adding acetic acid to the growth medium in response to an increase in pH resulting from the utilisation of acetic acid/acetate by *C. cohnii*.

Ratledge et al. (2003) also patented a method of culturing *C. cohnii* or microorganisms derived from it in a medium containing propionic acid that is said to cause an increase in the ratio of "cyst" to "swimmer" forms of the microorganism. The non-motile cysts are believed to accumulate greater levels of lipid, including docosahexaenoic acid, than the swimmer form of the organism. Moreover, the cysts do not expend energy in "swimming".

Final applications

Crypthecodinium cohnii can accumulate a high fraction of DHA with only trivial amounts of other PUFAs, which makes the DHA purification process from this microorganism very attractive, particularly for pharmaceutical applications, since the inclusion of PUFAs as a drug component requires its purification to over 95%. Senanayake and Shahidi (2000) reported a method for DHA concentration from the oil extracted from *C. cohnii* cells with a DHA enrichment from 47.1 to 97.1% with a process yield of 32.5% of the mass of the original algal oil. Mendes et al. (2007b) reported an alternative method of DHA purification from *C. cohnii* wet biomass, where a high DHA fraction (99.2% of TFA) was obtained. Such a process may be an economic alternative method of DHA purification since the extraction step was carried out from wet biomass rather than from lyophilized cells or extracted oil, time-consuming steps that are usually used in the traditional methods of DHA extraction and purification (Grima et al. 1993).

Single cell edible oil containing DHA can be used in infant formulas and baby foods, pharmaceutical products and dietary supplements (in the form of gelatine capsules) (Kyle et al. 1991). The Martek Corporation has patented the blending of its DHA from *C. cohnii* and sells it primarily for use in infant formulas. The company has entered into license agreements with 15 infant formula manufacturers which represent more than 70% of the world's wholesale infant formula market, including Mead Johnson Nutritionals (Bristol-Myers Squibb) among others. Formulas containing Martek's DHA oil are now available in more than 60 countries worldwide, including the United States, United Kingdom, Mexico, China, and Canada.

The residual biomass (i.e. cell carcasses that remain after fatty acids have been extracted from lysed cells) can be used as an animal feed, containing as it does about 35–40% protein, 8–10% ash and 45–50% carbohydrates (Kyle et al. 1991, 1998). Because of this high protein content and the elevated levels of DHA, the whole biomass paste can be

used for aquaculture (e.g., shrimp, oysters, fish) feed (Kyle et al. 1991).

Heterotrophically grown algae and their residual materials from an industrial oil extraction process were used as components in marine larval and broodstock diets. *Crypthecodinium* sp. phospholipid extract and meal, used to enrich rotifers and *Artemia* nauplii, produced higher levels of DHA and higher DHA/EPA ratios in these zooplankters. In addition, a 60% replacement of menhaden oil with algal oil and meal in striped bass broodstock diets resulted in a similar growth increase to that obtained with standard commercial diets. These findings demonstrate the potential of single cell heterotrophs as a partial substitute or replacement for fish-based ingredients in aquaculture diets (Harel et al. 2002). Fish and shrimp farming is rapidly growing, and DHA is an essential fatty acid for the growth of juvenile fish. The introduction of DHA from microalgae rather than from fish meal and oil, results in an additional benefit as microalgae are a completely renewable and contaminant-free resource.

The residual microalgal biomass can also be mixed with a combination of brewer's yeast, a grain product such as whole wheat, and burnt residues that collect on the interior walls of drying vessels used for spray-drying of yeast extracts. These ingredients are mixed with water to form a dough-like substance, which is made into a desired shape for dog, cat, or other pet treats. The dough is then cooked to form a pleasant-smelling biscuit-type treat which dogs and cats find highly appealing. Algal biomass can also be incorporated into "flavor or palatability enhancer" additives that are coated into surfaces of pelleted or extruded chunks, to prepare a flavored dog or cat food. Using algal of fungal biomass in the manner eliminates a noxious and foul-smelling waste problem for manufacturers of nutrition supplementes. In addition, pet foods created in this manner contain quantities of DHA, a nutrient that is highly valuable in food for dogs and cats (Criggal et al. 2002). This invention relates to foods for mammalian companion animals, including cats, dogs, ferrets, and pot-bellied pigs. Although it may be also useful for livestock, poultry, or other farm animals, or for aquaculture (Criggal et al. 2002; Calado et al. in press).

As *C. cohnii* is non-photosynthetic it is potentially useful as a biochemical tool for the characterization of the enzymes involved in the production of 22:6ω-3 without the complication of the substrate transfer between chloroplast and non-chloroplast lipids. *C. cohnii* possesses all the enzymes necessary for de novo synthesis of 22:6ω-3 and is therefore an ideal candidate for use in studies on the pathways and enzymology of fatty acid desaturation and elongation (Henderson and Mackinlay 1991).

Microalgal bioprocesses for ω-3 PUFAs production, namely DHA, has been considered of economic interest. *Crypthecodinium cohnii* is a valuable source for DHA production, and several aspects of the process can be further improved, including strain selection, medium composition, carbon-source feeding strategy and control. Genetic engineering of this microorganism may potentially lead to even higher productivity at lower costs.

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